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Line Probe Assay for Rapid Detection of Drug-Selected Mutations in the Human Immunodeficiency Virus Type 1 Reverse Transcriptase Gene

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Upon prolonged treatment with various antiretroviral nucleoside analogs such as 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyinosine, 2′,3′-dideoxycytidine, (−)-β-L-2′,3′-dideoxy-3′-thiacytidine, and 2′,3′-didehydro-3′-deoxythymidine, selection of human immunodeficiency virus type 1 (HIV-1) strains with mutations in the reverse transcriptase (RT) gene has been reported. We designed a reverse hybridization line probe assay (LiPA) for the rapid and simultaneous characterization of the following variations in the RT gene: M41 or L41; T69, N69, A69, or D69; K70 or R70; L74 or V74; V75 or T75; M184, I184, or V184; T215, Y215, or F215; and K219, Q219, or E219. Nucleotide polymorphisms for codon L41 (TTG or CTG), T69 (ACT or ACA), V75 (GTA or GTG), T215 (ACC or ACT), and Y215 (TAC or TAT) could be detected. In addition to the codons mentioned above, several third-letter polymorphisms in the direct vicinity of the target codons (E40, E42, K43, K73, D76, Q182, Y183, D185, G213, F214, and L214) were found, and specific probes were selected. In total, 48 probes were designed and applied on the LiPA test strips and optimized with a well-characterized and representative reference panel. Plasma samples from 358 HIV-infected patients were analyzed with all 48 probes. The amino acid profiles could be deduced by LiPA hybridization in an average of 92.7% of the samples for each individual codon. When combined with changes in viral load and CD4$^+$ T-cell count, this LiPA approach proved to be useful in studying genetic resistance in follow-up samples from antiretroviral agent-treated HIV-1-infected individuals.

Reverse transcriptase (RT) inhibitors such as the nucleoside analogs 3′-azido-3′-deoxythymidine (AZT; zidovudine), 2′,3′-dideoxyinosine (ddI; didanosine), 2′,3′-dideoxycytidine (ddC; zalcitabine), (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC; lamivudine), and 2′,3′-didehydro-3′-deoxythymidine (d4T; stavudine) are the nucleosides currently approved for the treatment of advanced human immunodeficiency virus (HIV) type 1 (HIV-1) infections (2, 19). All these compounds act in a similar way, namely, as chain terminators of the RT reaction after phosphorylation by intracellular kinases (5, 20). Unfortunately, resistance to these drugs has been increasingly observed (10, 15, 17, 18, 21). Upon prolonged treatment with these nucleoside analogs, viral variants having the possibility of escaping the inhibitory effects of these antiviral agents are selected. Resistant viral strains show nucleotide changes in the RT gene (17, 18), resulting in amino acid (aa) changes that lead to gradually increasing resistance. Among these changes, aa positions 41 (M to L), 69 (T to D), 70 (K to R), 74 (L to V), 184 (M to V), and 215 (T to Y or F) are known to be of particular importance (13). Mutations at aa's 65, 67, 75, and 219, and the more recently described aa changes at codons 62, 75, 77, 116, and 151 encoding multidrug resistance (7) may also be of equal significance. A detailed overview of genotypic resistance profiles and their corresponding phenotypic resistance consequences is available (13).

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Although anti-HIV therapy with nucleoside analogs was introduced several years ago, the optimal algorithm for efficient treatment remains unclear. Currently, the best prognostic markers of survival for HIV-infected individuals undergoing antiviral treatment are obtained by monitoring the changes in viral load and CD4$^+$ T cells (14). The appearance of one or several of the RT variants during antiviral treatment (genotypic resistance) with respect to disease progression and clinical deterioration is generally not interpreted in conjunction with the parameters listed above. For example, in vitro studies have shown that the effect of AZT resistance mutations can be suppressed after the appearance of the 3TC-selected M184V mutation (1, 11, 21). However, the clinical influence of this genetic combination needs further evaluation, especially in long-term treatment studies. In order to gain a better insight into the mechanisms of genotypic drug resistance and HIV biology, a genotyping line probe assay (LiPA) was developed. This assay allows for the rapid detection of variations at those aa positions conferring resistance to antiviral drugs (see Tables 1 to 6). The principle of the assay is based on reverse hybridization of a biotinylated PCR fragment of the relevant part of the HIV RT with short, immobilized oligonucleotides (22, 23). The latter hybrid can then be detected via a biotin-streptavidin coupling with a colorimetric system. The selection of these probes is described herein, and their applicability is demonstrated by analyzing plasma samples from European and American HIV-1-infected individuals.

Genotypic resistance of the HIV-1 RT gene can be determined by means of several molecular biology applications: Southern blotting (16), primer-specific PCR (10), PCR-ligase

detection reaction (4), RNase A mismatch (6), hybridization against labeled probes (3), point mutation assay (9), and the LiPA described here. The relative merits or disadvantages of deriving genotypic data in the absence of phenotypic data (8) are still open questions. The development of rapid, sensitive, and simple genotyping and phenotyping tests can increase the understanding of that relation.

MATERIALS AND METHODS

Plasma sample collection. Plasma samples were taken from HIV-1-infected patients and were stored at −20°C until use. Patients were treated with AZT, ddI, ddC, d4T, 3TC, or several combinations of these drugs. The European serum samples were randomly selected. For the U.S. serum sample collection, only the first sample from a follow-up series was taken. Some of these U.S. patients were treated, while others were not.

HIV RNA preparation, cDNA synthesis, and PCR. HIV RNA and cDNA preparation was identical to that described for the hepatitis C virus (HCV) (22, 23). Briefly, 50 μl of plasma was mixed with guanidinium-phenol. After lysis and denaturation, $CHCl_3$ was added to obtain phase separation, and nucleic acids were precipitated from the aqueous phase with isopropanol and were collected by centrifugation. The RNA pellet was dissolved in a random primer solution [$pd(N)_6$; Pharmacia, Brussel, Belgium]. cDNA synthesis occurred with avian myeloblastosis virus RT (Stratagene, La Jolla, Calif.) at 42°C. The HIV RT gene was then amplified in the following mixture: 5 μl of cDNA, 4.5 μl of 10× *Taq* buffer, 0.3 μl of 25 mM (each) deoxynucleoside triphosphates, 1 μl (10 pmol) of each PCR primer, 38 μl of H_2O, and 0.2 μl (1 U) of *Taq* (Stratagene). The annealing temperature was set at 57°C, extension was at 72°C, and denaturation was at 94°C. Each step of the cycle took 1 min. The outer PCR contained 40 cycles, and the nested reaction contained 35 cycles. The annealing temperature seemed to be crucial (57°C). At 55°C, a second aspecific amplicon of approximately 1,500 bp was generated, and at 59°C the amount of specific fragment decreased markedly. Nested PCR products were analyzed on agarose gels, and only clearly visible amplification products were used in the LiPA procedure. Quantification of viral RNA was done by the HIV Monitor test (Roche, Brussels, Belgium). The detection limit in these experiments was between 150 and 200 viral copies per ml of plasma.

Primers. PCR primers outside the target regions were chosen for probe design; their sequences were based on published HIV-1 genotype B information. The amplified region located inside the nested primers covered the RT gene from codon 29 to codon 220. The primers had the indicated sequences: outer sense primer RT-9, 5′-bio-GTACAGTATTAGTAGGACCTACACCTGTC-3′; nested sense primer RT-1, 5′-bio-CCAAAAGTTAAACAATGGCCATTGAC AGA-3′; nested antisense primer RT-4, 5′-bio-AGTTCATAACCCATCCAAA G-3′; and outer antisense primer RT-12, 5′-bio-ATCAGGATGGAGTTCATA ACCCATCCA-3′. With the current primer combination, the corresponding RT region of the HIV-1 genotype A, C, D, and F clade could also be amplified, but with a reduced sensitivity.

In principle, any biotinylated PCR product covering the RT region from codon 29 to codon 220 can be used in this assay without restrictions to the amplicon size. However, since the assay was optimized for end-standing biotin groups, it is advisable to keep this amplicon as short as possible. The indicated PCR primers were carefully selected accordingly.

Probes. Probes were designed to cover different polymorphisms and drug-selected mutations. In principle, only those probes that discriminated between a single nucleotide variation were retained. For certain polymorphisms at the extreme ends of the probe, cross-reactivity was tolerated. Specificity was reached for each probe individually after considering the percent G+C content, probe length, final concentration, and hybridization temperature.

In total, 48 specific oligonucleotide probes, able to detect 44 sequence motifs in the HIV-1 RT gene, were selected (Tables 1 to 6). This selection can be split into four subsequent steps. (i) A total of 35 probes were based on sequence motifs present in the National Center for Biotechnology Information (NCBI; Bethesda, Md.) nucleotide sequence database. All HIV-1 genome entries were retrieved and analyzed one by one. Only those entries displaying nonambiguous sequence information in the vicinity of the codons mentioned above were retained for further interpretation. For the design of relevant probes, only those database motifs that systematically returned (highly prevalent motif) were included, while scattered mutations which were found randomly (low prevalent motif; data not shown) were excluded. On the basis of database sequences, eight probes for codon 41 (91.6% of all entries), seven for codons 69 and 70 (86.2%), two for codons 74 and 75 (90.4%), five for codon 184 (96.6%), 11 for codon 215 (94.1%), and 2 for codon 219 (88.2%) were selected. Four probes (probes 41w15, 70w8, 215w29, and 215w27) are, in fact, redundant, because they detect identical sequence motifs covered by other probes. The locations of these redundant probes are slightly different with respect to their counterpart with an identical sequence. These probes can prevent negative results which might otherwise appear as a consequence of random mutations in the probe target area and can therefore increase the sensitivity of recognition. (ii) By analyzing European and U.S. plasma samples, another eight motifs not predicted in the database appeared. The corresponding probes (probes 41w20, 41m12, 70m13, 74w9, 74m6, 74m12, 184w24, 215m49) were designed. (iii) Another four probes (probes 41m11, 215m50, 219m7, and 219m9) were optimized because their sequence motif was found in recombinant clones retrieved from plasma (see the section on the reference panel below). It is assumed that these motifs exist at an extremely low frequency in the viral quasispecies, remaining undetectable by direct detection methods, but becoming apparent after cloning. (iv) The sequence motif of probe 215m13 was generated in recombinant clones by site-directed mutagenesis (data not shown). The rationale for this was to determine whether the sequence combination of codon Y215 (TAC) can occur in combination with L214 (CTT) in vivo.

Reference panel. Selected PCR products, amplified without 5′ biotin primers, were cloned into the pretreated *Eco*RV site of the pGEMT vector (Promega Corp. Benelux, Leiden, The Netherlands). Recombinant clones were selected after α-complementation and restriction fragment length analysis and were then sequenced with plasmid primers and internal HIV RT primers. Biotinylated fragments were directly sequenced with the ABI Prism dye terminator protocol (Perkin-Elmer, ABD, Foster City, Calif.) by using the amplification primers. Alternatively, nested PCR was carried out with analogs of the RT-4 and RT-1 primers, in which the biotin group was replaced with the T7 and SP6 primer sequences, respectively. These amplicons were than sequenced with an SP6 and T7 dye primer procedure.

LiPA strip preparation. Optimized probes were provided enzymatically with a poly(T) tail by using terminal deoxynucleotidyl transferase (Pharmacia) under standard reaction conditions and were purified via precipitation. Probe pellets were dissolved in standard saline citrate buffer and were applied as horizontal parallel lines on a membrane strip. Control lines for amplification (probes 5′-C CACAGGGATGGAAAG-3′ and 5′-GATCTGACTTAGAAATAG-3′) and conjugate incubation (biotinylated DNA) were applied alongside. After fixation of the probes, membranes were sliced into 4-mm strips.

LiPA test performance. Equal amounts (10 μl) of biotinylated amplification products and denaturation mixture (0.4 N NaOH and 0.1% sodium dodecyl sulfate) were mixed, followed by an incubation at room temperature for 5 min. Following this denaturation step, 2 ml of prewarmed hybridization buffer (containing Tris-HCl, sodium dodecyl sulfate, and standard saline citrate) was added together with a membrane strip, and hybridization was carried out at 39°C for 30 min. The hybridization mixture was then replaced with a stringent wash buffer, and washing occurred first at room temperature for 5 min and then at 39°C for another 10 min. Buffers were then replaced for the streptavidin-alkaline phosphatase conjugate incubations. After 30 min of incubation at room temperature, the conjugate was rinsed away and was replaced with the substrate components for alkaline phosphatase, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich, Bornem, Belgium). After a 30-min incubation at room temperature, the probes in which hybridization occurred became visible as a purple-brown precipitate at these positions.

Nucleotide sequence accession numbers. Sequence information was submitted to GenBank and is available under accession numbers L78133 to L78157 (for the 18 PCR fragments cloned in pGEMT, accession numbers L78139 to L78156, respectively; for the 7 PCR fragments amplified from plasma virus, accession numbers L78133 to L78138 and L78157, respectively).

RESULTS

The HIV-1 RT gene and reference panel PCR. A total of 96% of the European and U.S. HIV-1-positive plasma samples, stored appropriately (at −20°C) without repeated freezing-thawing cycles, were positive by PCR (data not shown). During the probe selection procedure, a total of 25 PCR fragments with the desired target polymorphisms and mutations were retained as a reference panel and were eventually cloned in pGEMT, and both strands were sequenced. Biotinylated PCR products from this panel were used to test and optimize repeatedly the selected probes for specificity and sensitivity.

Probe specificity and sensitivity. The 48 selected probes were applied separately on LiPA strips, and each strip was incubated with a biotinylated PCR fragment generated from the reference panel or directly from virus in plasma (Fig. 1a to f). The reactivities of these probes were concordant with the nucleotide sequences. At the current stage of the ongoing probe development, cross-reactivities were observed only for probe 41w19 (Fig. 1a, lane 9) and probe 70m3 (Fig. 1b, lane 8), with extremely rare sequence motifs 41m12 (prevalence less than 0.3%) and 70m16 (not experimentally found), respectively.

Performance characteristics. Since this assay works with any biotinylated PCR fragment, the limitation of the technique is

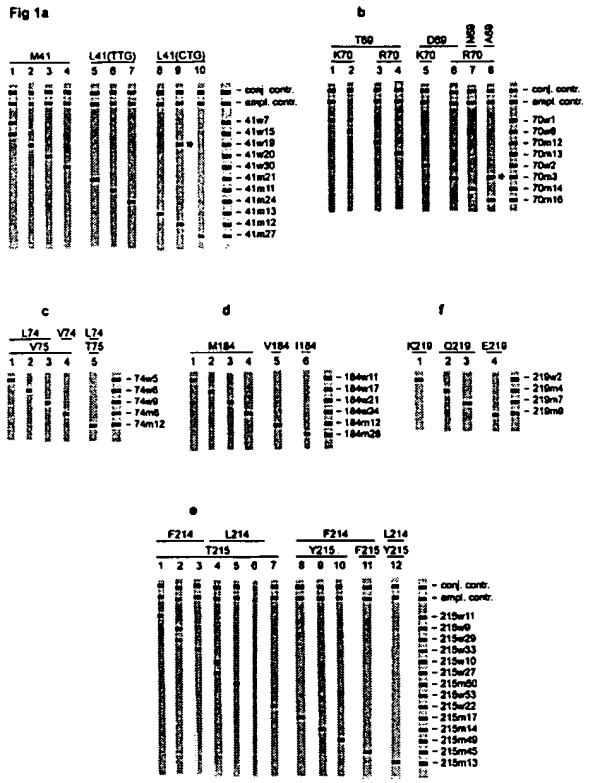

FIG. 1. Reactivities of the selected probes, immobilized on LiPA strips, with reference material. (a) Probes for codon 41; (b) probes for codons 69 and 70; (c) probes for codons 74 and 75; (d) probes for codon 184; (e) probes for codon 215; (f) probes for codon 219. The position of each probe on the membrane strip is shown at the right of each panel. The sequences of the relevant parts of the selected probes are given in Tables 1 to 6. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Tables 1 to 6. For several probes, multiple reference panel possibilities are available, but only one relevant accession number is given each time. *, cross-reactive probes. On top of the strips, the aa's at the relevant codon, as derived from the probe reactivity, are indicated.

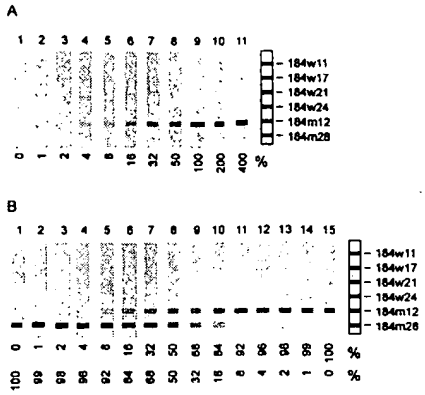

FIG. 2. Performance characteristics of the LiPA for codons 184V and 184I. (A) Strips were incubated with an increasing amount of biotinylated material from reference panel clone L78142, the amount of material is indicated beneath each strip (100% is considered the standard amount of amplicons used in routine diagnostic tests). The positions of the probes on the strips are indicated on the right; names correspond to the sequences in Table 4. (B) Artificial mixture of reference panel clones L78142 and L78148. The amount of materials used to obtain these reactivities is indicated.

only dependent on the variability of the virus and on the sensitivity of the RT-PCR procedure. We used as an RNA input standard the detection limit in the Amplicor (Roche) procedure, which was set at 150 to 200 viral copies per ml of plasma. Our in-house RT-PCR methodology was able to amplify samples containing less than 1,000 copies per ml, which consequently resulted in fully interpretable LiPA results (see Fig. 3 for data for patient B).

Figure 2 shows some further characteristics of the sensitivity of detection. We prepared two amplification products at comparable concentrations (100% in Fig. 2 corresponds to approximately 250 ng of the PCR fragment) from the reference panel (L78142 and L78148). These samples were reactive with the probes for codons 184V and 184I, respectively. Figure 2A shows the sensitivity of detection in a dilution experiment. A weak but clearly distinguishable signal is already present at a concentration of 4% (10 ng) to 8% (20 ng) of the normal amount of amplicons used in LiPA experiments. This signal increases with an increasing amount of material, but no aspecific reaction occurred, even in heavily overloaded settings (Fig. 2A, lane 11; 1 μg). In a second experiment (Fig. 2B), mixtures of both amplicons were prepared. Again in this experiment, amplicons were clearly visible and were specifically reactive at a concentration of 4% or higher. Comparable results were obtained for codons at other positions (data not shown).

Analysis of European and U.S. serum samples. The results obtained with these 48 probes with 306 European and 52 U.S. plasma samples from HIV-1-infected individuals are summarized in Tables 1 to 6. Because of the inclusion of probes for motifs not reported in the NCBI database, the percentage of motif recognition in these plasma panels is generally higher than could be predicted from the database. At each relevant codon, between 82.4% (codon 219 in European samples) and 100% (codon 41 in U.S. samples) of the variants could be assigned unambiguously (average, 92.7%). In several plasma samples (n = 38), we found sequence motifs which were not predicted from the database. This was particularly apparent at codon 74 (74w9) and at codon 41 (41w20).

Analysis of follow-up samples from patients. We selected follow-up samples from three patients and analyzed the viral genotype with the 48 LiPA probes. The LiPA genotyping result together with the viral load and $CD4^+$ T-cell count are presented in Fig. 3. For all three patients, a wild-type virus (i.e., M41-T69-K70-L74-V75-M184-F214-T219-K219) strain was found in the sample collected before antiretroviral treatment. Only codon positions where changes occurred after treatment are presented in Fig. 3.

From patient A, 11 plasma samples were analyzed, with the first sample being collected 2 weeks before the start of therapy. LiPA revealed that before treatment, in a T215 context (all variants having threonine at codon 215), two variants at codon position 213 were predominantly present (GGG and GGA detected by probes 215w11 and 215w9/215w29, respectively). From week 50 to week 81, a mixture of T215 and Y215 could be detected. Both mutants with variations at codon 213 were also represented among the selected mutants with resistance genotypes (probes 215m17 and 215m14 were both positive). From week 94 onward, only Y215 mutant virus could be detected. A nearly identical genoconversion at codon 41 was observed, with the detection of mixtures (M41 and L41) from week 81 to week 111; from week 126 onward, only L41 could be found (data not shown). $CD4^+$ T-cell values were highly variable, but a continuous decrease was apparent (P = 0.019;

TABLE 1. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 38 to 43

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 191/m = 25)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	TGTACAGAAATGGAAAAG	CTEMEK						
41w7	----------	----	122 (62.9)	237	35	11	a, 1	L78149
41w15[f]	----------	-----	118	230	38	9	a, 1	L78149
41w19	-----G------	----	5 (2.6)	10	2	2	a, 2	L78156
41w20	--G--------A	----	0	6	0	1	a, 3	L78157
41w30	--------G---	----	1 (0.5)	8	6	1	a, 4	L78154
41m21	------T--------	--L--	18 (9.4)	37	7	2	a, 5	L78136
41m11	--GT--------	-L--	0	0	0	1	a, 6	L78140
41m24	---T-----G--	-L-E	12 (6.3)	1	2	1	a, 7	L78144
41m13	---C--------	-L--	14 (7.3)	21	3	1	a, 8	L78139
41m12	--GC--------	-L--	0	1	0	1	a, 9	L78155
41m27	---C-----G--	-L-E	3 (1.6)	0	1	1	a, 10	L78137
Total (%)[g]			175 (91.6)	95.1	100	88		

[a] Consensus sequence covering the vicinity of the aa motifs conferring drug resistance; the nucleotide consensus presented does not provide the exact probe sequence, but only that region that represents the required specificity motif indicated by the consensus aa.
[b] Prevalence of the indicated motifs in the NCBI nucleic acid sequence database. n, total amount of consensus and variant sequences that were retrieved; m, amount of different motifs present in the n amount of sequences.
[c] RP, reference panel indicating the number of times a certain motif was cloned.
[d] The indicated number corresponds to a lane in Fig. 1.
[e] Accession number indicating the location of the corresponding reference panel clone in the NCBI sequence database.
[f] Redundant probe having the same sequence motif as the previous probe, but with a slightly different location compared to the consensus sequence.
[g] The total percentage for European and U.S. samples is not the sum of the probe reactivities, but a result of the complete interpretations for these codons. This is due to the fact that some sera showed mixed (wild type and mutant) reactivities.

linear regression analysis). Viral load also decreased initially. However, the direct response to the treatment might have been missed in this follow-up series, since the first sample after the start of the treatment was obtained at 32 weeks. From then on, the viral load increased.

Patient B was treated with combination AZT-3TC therapy from week 2 onward. At week 10, a mixture of M184 and V184 could be detected. From week 14 on, only V184 was present. $CD4^+$ T-cell counts increased nearly 2.5-fold, with the highest level found at week 10. Viral load decreased spectacularly by 3 log units. From week 10 onward, however, a slight but steady increase was noted. The decrease in $CD4^+$ T cells and the increase in viral load coincided with the appearance of the V184 motif. AZT resistance codons were not yet apparent by week 23.

Patient C was followed for 55 weeks. AZT treatment started at week 10, followed by a supplemental ddC treatment from week 20 onward. The first sample was found to be reactive with probe 215w9/w29 (F214 T215 = TTTACC), but trace amounts of reactivity with 215w53 (L214 T215 = TTAACC) could be detected as well, indicating the presence of at least two variants at that time. From week 19 onward, the codon L214 (TTA)

TABLE 2. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 68 to 72

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 354/m = 32)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	AGTACTAAATGGAGA	STKWR						
70w1	----------	-----	224 (63.3)	230	39	13	b, 1, 2	L78147
70w8[f]	----------	----	208	210	38	11	b, 2	L78144
70m12	-------G--------	--R--	37 (10.5)	46	6	4	b, 3	L78148
70m13	-----A-G--------	--R--	0	0	1	2	b, 4	L78133
70w2	---GA----------	-D---	25 (7.1)	4	4	2	b, 5	L78136
70m3	GA--G--------	DR--	10 (2.8)	3	1	0	b, 6	Pending
70m14	----A--G----	-NR-	7 (2.0)	4	5	2	b, 7	L78154
70m16	---G---G----	-AR-	2 (0.6)	0	0	1	b, 8	L78150
Total (%)[g]			305 (86.2)	91.8	94.2	96		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *f* to Table 1.
[g] See note *g* to Table 1.

TABLE 3. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 72 to 77

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 364/m = 20)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	AGAAAATTAGTAGATTTC	RKLVDF						
74w5	----------	------	320 (87.9)	264	48	16	c, 1	L78150
74w8	----------C---	------	9 (2.5)	34	1	2	c, 2	L78147
74w9	-----G-----G---	------	0	17	3	2	c, 3	L78137
74m6	------G--------	--V---	0	5	0	3	c, 4	L78149
74m12	------AC-------	--T---	0	1	1	1	c, 5	L78136
Total (%)[f]			329 (90.4)	93.5	98.1	96		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *g* to Table 1.

motif became more important. At week 42, the first sign of genotypic resistance could be detected by the presence of a F214 Y215 motif (TTTTAC). Finally, at week 55, only the F214 Y215 motif could be detected. The L214 (TTA) motif disappeared completely. At week 42, a mixture (K and R) at codon 70 was present, but at week 55, only R70 could be detected. Also at week 55, a mixture of codon 219 motifs (K and E) was found (data not shown). The patient's $CD4^+$ T-cell count increased initially, with a maximal effect during AZT monotherapy peaking at week 21. From then on, a continuous decrease was observed. However, 10 weeks of AZT treatment did not result in a drop in viral load, since the values at weeks 16 and 19 were nearly unchanged. It was only after start of the combination therapy (week 20) that the viral load dropped by 1.67 logs. The rise in $CD4^+$ T-cell count may be the consequence of the drug itself and not drug-induced protection (12). Phenotypic testing should be performed to determine whether the L214 T215 motif in this specific genetic background confers a certain level of resistance.

DISCUSSION

By adapting the previously designed HCV genotyping LiPA technology (22, 23) for the HIV RT gene, the LiPA format permits the rapid and simultaneous detection of wild-type and drug-selected variants associated with the genotypic resistance for AZT, ddI, ddC, d4T, and 3TC. The combination of the selected probes provides information about the genetic constitution of the RT gene in the vicinity of codons 41, 69, 70, 74, 75, 184, 215, and 219 at the nucleotide level and, hence, also at the deduced protein level. Essentially, the biotinylated RT PCR product is hybridized against immobilized specific oligonucleotides (Tables 1 to 6) which are directed against the indicated codon variabilities. Following this reverse hybridization, the oligonucleotide-biotinylated PCR strand is recognized by the streptavidin-alkaline phosphate conjugate, which then in turn converts the alkaline phosphate substrate into a purple-brown precipitate.

Using this assay, we studied the specificities and reactivities of 48 probes covering six different regions. This combination

TABLE 4. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 182 to 185

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 322/m = 12)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	CAATACATGGAT	QYMD						
184w11	----------	----	285 (88.5)	267	46	18	d, 1	L78147
184w17	--G-------	----	16 (5.0)	9	4	3	3, 2	L78137
184w21	-----T------	----	6 (1.9)	4	2	1	d, 3	L78145
184w24	----------C	----	0	1	0	1	d, 4	L78144
184m12	------G-----	--V-	1 (0.3)	8	0	1	d, 5	L78142
184m28	--------A---	--I-	3 (0.9)	0	0	1	d, 6	L78148
Total (%)[f]			311 (96.6)	93.8	98.1	100		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *g* to Table 1.

TABLE 5. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 212 to 218

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motif in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 321/m = 36)[b]	Europe (n = 306)	United States (n = 52)	RP (n = 25)[c]		
	TGGGGATTTACCACACCAGAC	WGFTTPD						
215w11	G----------	----	9 (2.8)	15	3	2	e, 1	L78146
215w9	----------	-----	142 (44.2)	178	24	3	e, 2	L78141
215w29[f]	----------	----	142	105	16	3	e, 2	L78141
215w33	-----C------	----	9 (2.8)	8	4	1	e, 3	L78154
215w10	C----------	L---	25 (7.8)	10	0	2	e, 4	L78150
215w27[f]	------C-----	--L-	25	14	0	2	e, 4	L78150
215m50	--GC--------	-L--	0	0	0	1	e, 5	L78145
215w53	-----A------	-L--	1 (0.3)	1	3	1	e, 6	L78138
215w22	--T---------	----	3 (0.9)	10	2	1	e, 7	L78134
215m17	------TA----	--Y-	88 (27.4)	50	12	7	e, 8	L78144
215m14	--G---TA----	--Y-	24 (7.5)	24	1	1	e, 9	L78149
215m49	--G---TAT---	--Y-	0	2	0	2	e, 10	L78148
215m45	---TT------	-F--	1 (0.3)	16	0	1	e, 11	L78135
215m13	---C--TA----	-LY-	0	0	0	2	e, 12	L78155
Total (%)[g]			302 (94.1)	92.8	90.4	96		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *f* to Table 1.
[g] See note *g* to Table 1.

should allow for the reliable detection of most of the genetic resistance-related codon combinations observed to date. Mutations occasionally occurring in the vicinity of the target codons, which were not taken into consideration during probe design, may eventually prevent hybridization of the probes to a particular target region. This problem is partially solved by the redundancy of probes at the most important codons. Results obtained with 358 HIV-1-infected plasma samples showed that, depending on the codon position under investigation, between 82.4 and 100% of the combinations could be detected (average, 92.7%). It is important to mention here that the assay was developed for the detection of resistance of HIV-1 genotype B, which is found predominantly in Europe and the United States, and only limited information is available about the outcome of this assay with other HIV-1 genotypes. Since the amplification primer combination is more or less universal for all the HIV-1 isolates, some of the indeterminate results may well be due to the presence of non-genotype B virus strains.

Because of the large numbers of variables that need to be included in the selection of specific probes (temperature of hybridization, ionic strength of hybridization buffer, length of the probe, G+C content, strand polarity), some of the probes might occasionally show weak cross-reactivity with related but hitherto unreported sequences. In our experience, this has never influenced the interpretation at the deduced aa level. In the current selection of probes, all except two (41w19 and 70m3) were retained on the basis of 100% specificity: as soon as one nucleotide differs in the probe area, hybridization is abolished. Further ongoing fine-tuning of these probes can

TABLE 6. Prediction and prevalence of LiPA probe reactivity based on natural and drug-selected HIV-1 RT gene variability for codons 217 to 220

Probe	Consensus sequence[a]		Prevalence (no. [%] of samples) of variant motifs in:				Panel, lane in Fig. 1[d]	Accession no.[e]
	Nucleic acid	aa	Database (n = 204/m = 12)[b]	Europe (n = 34)	United States (n = 52)	RP (n = 26)[c]		
	CCAGACAAAAAA	PDKK						
219w2	----------	----	179 (87.7)	26	42	18	f, 1	L78144
219m4	------C-----	--Q-	1 (0.5)	2	4	2	f, 2	L78135
219m7	-----TC-----	--Q-	0	0	0	1	f, 3	L78133
219m9	------G-----	--E-	0	0	0	1	f, 4	Pending
Total (%)[f]			179 (88.2)	82.4	82.7	84.6		

[a] See note *a* to Table 1.
[b] See note *b* to Table 1.
[c] See note *c* to Table 1.
[d] See note *d* to Table 1.
[e] See note *e* to Table 1.
[f] See note *g* to Table 1.

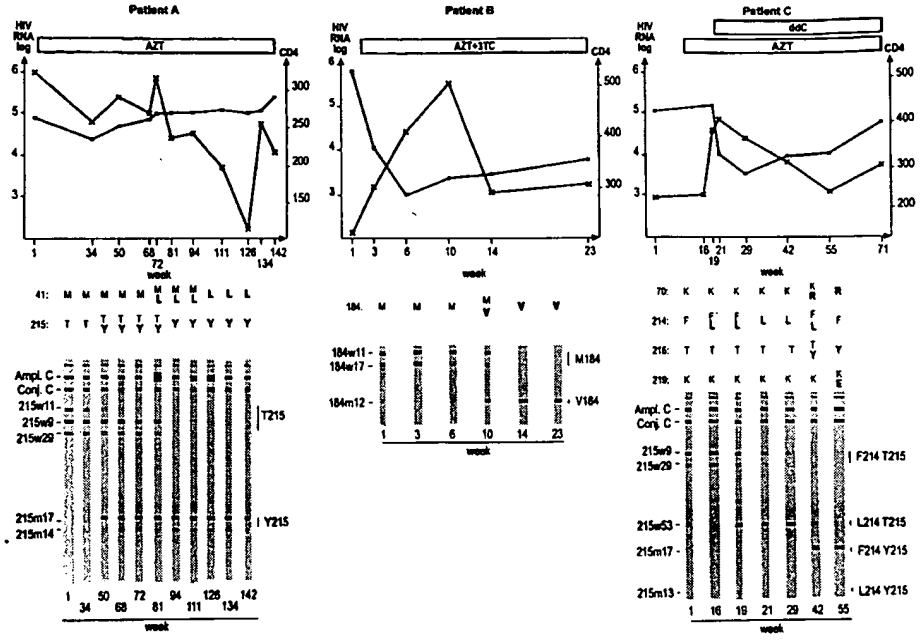

FIG. 3. Clinical and virological features detectable in follow-up samples from three patients, patients A to C. All three patients were infected with an HIV-1 strain showing the M41-T69-K70-L74-V75-M184-F214-T215-K219 genotype (wild-type pattern). Fluctuations between plasma HIV RNA copy numbers (log_{10} copies/ml) (■) and $CD4^+$ T-cell count (×) are given as a function of time in the panels on the top. The different treatment regimens and the period of treatment are also indicated. The sequences in the middle indicate the changes that appeared during the treatment period and that could be scored with the LiPA probes: for patient A at codons 41 and 215, for patient B at codon 184, and for patient C at codons 70, 214, 215, and 219. The corresponding LiPA strips for a subset of the aa changes are shown at the bottom. LiPA probes are indicated on the left, and the aa interpretation is indicated at the right of each panel. Ampl. C, amplification control; Conj. C, Conjugate control.

enhance the required specificity. For other weak cross-reactivities, the following interpretation rule is generally followed: if cross-reactivity is occurring with a cloned amplification product, this might be due to technical conditions (illustrating the necessity for stringent compliance with the described procedure); if the same result is obtained with amplification products derived from a plasma sample, the possibility of a mixture should be considered as well.

Several assays for the detection of wild-type and drug-selected mutations in the HIV RT gene have been described. These include Southern blotting (16), primer-specific PCR (10), PCR-ligase detection reaction (4), RNase A mismatch cleaving (6), point mutation assay (9), and hybridization against enzyme-labelled probes (3). The general advantage of the LiPA and other genotypic assays is the speed with which results are obtained compared to the speed with which the results of phenotypic assays are obtained. The particular advantage of our test is its multiparameter (in this particular case, multicodon) format. Moreover, the assay can easily be extended not only for the screening of the other RT codons but also for the screening of proteinase codons associated with resistance (13). As illustrated in Fig. 2, mixtures of different sequence variants can be detected easily. The detection limit for these mixtures is dependent on the sensitivity of the probes; but with amplicon concentrations ranging from 5 ng (2%) to 20 ng (8%), or an average of 10 ng (4%; Fig. 2), reliable staining patterns were observed. This is comparable to the experiences encountered during the development of the HCV genotyping LiPA (23). Our sequencing protocol has not yet provided the same degree of sensitivity in detecting such mixtures.

Accompanying polymorphisms in the vicinity of the target codons are found with a rather high prevalence in wild-type virus strain sequences, but not in mutant strain sequences. A partial list of such combinations is hereby presented: codon V74 (GTA) without polymorphism at codon 73, 75, or 76; codon V184 (GTG) without codon Q182 (CAG); and codon F215 (TTC) without F214 (TTC or TTA) or L214 (CTT). The most intriguing example is the following: L214 T215 (CTTA CC) is predicted for approximately 7.8% of the wild-type sequences. The corresponding motif, L214 Y215 (CTTTAC), apparently does not exist in virus from plasma. From the example shown in Fig. 3c, it is clear that the selection of mutants is a very flexible and complex phenomenon. In this particular case, viruses having codon F214 were replaced by a L214 viral population in the AZT monotherapy period, but upon selecting for genotypic drug resistance at codon 215, the original F214 configuration was restored. The selection for the Y215 genotype prohibits the presence of an L214 genotype. Since no evidence has yet emerged that L214 confers resistance to antiretroviral compounds, the appearance of this special mutant during AZT monotherapy period is difficult to interpret and most likely depends on the genetic background. More research will certainly be necessary to clarify this issue. This includes phenotypic resistance determinations and site-directed mutagenesis experiments for verification. These experiments are in progress.

Since antiviral treatment can result in a marked extension of life expectancy for HIV-infected individuals, it is of utmost importance to find the best drug regimen for each individual. Therefore, monitoring of the magnitude and duration of the virus load and $CD4^+$ T-cell changes is a prerequisite (14). However, knowledge concerning the genetic constitution of the virus may also be an important factor in designing optimal treatment schedules. Optimization of therapies by making

good use of available information (viral load, $CD4^+$ T-cell count, and resistance profiles) has largely remained unexploited. If this was partially due to the complexity of screening for all the mutational events by the more conventional methods, the introduction of rapid genotypic and/or phenotypic assay systems should remove one key obstacle.

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Exhibit B

Guidance for Industry

Premarket Notifications [510(k)s] for In Vitro HIV Drug Resistance Genotype Assays: Special Controls

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.

Comments and suggestions regarding this draft document should be submitted within 60 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit comments to Dockets Management Branch (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. All comments should be identified with the docket number listed in the notice of availability that publishes in the *Federal Register.*

Additional copies of this draft guidance document are available from the Office of Communication, Training and Manufacturers Assistance (HFM-40), 1401 Rockville Pike, Suite 200N, Rockville, MD 20852-1448, or by calling 1-800-835-4709 or 301-827-1800, or by Fax 1-888-CBERFAX or 301-827-3844, or from the Internet at http://www.fda.gov/cber/guidelines.htm.

For questions regarding this draft document contact Andrew Dayton, M.D., Ph.D., (301) 827-0802.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research (CBER)
August 2001

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GUIDANCE FOR INDUSTRY

Premarket Notifications [510(k)s] for In Vitro HIV Drug Resistance Genotype Assays

This guidance document represents FDA's current thinking on special controls for HIV drug resistance assay premarket notifications [510(k)s]. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such approach satisfies the requirements of the applicable statutes and regulations.

I. INTRODUCTION

A. Purpose

We, the Center for Biologics Evaluation and Research (CBER), Food and Drug Administration (FDA) have issued this draft guidance to assist you, manufacturers and sponsors of HIV Drug Resistance Assays, to comply with the requirement of special controls for class II devices, if the HIV Drug Resistance Assay devices are reclassified from Class III. Designation of this guidance document as a special control would mean that you must establish that your device complies with either the specific recommendations of this guidance or some alternative control that provides equivalent assurances of safety and effectiveness [§513(f) (21 U.S.C 360c(f)]. You will help ensure the production of standardized, reliable, and reproducible tests for detecting HIV mutations known to be associated with HIV drug resistance, if you follow the recommendations in this document.

B. Definition

An HIV Drug Resistance Genotype Assay is an in vitro diagnostic device (IVD) intended for clinical laboratories to use in detecting HIV genomic mutations that confer resistance to specific anti-retroviral drugs, as an aid in monitoring and treating HIV infection.

C. Background

Clinically, HIV drug resistance testing has been shown to be useful for therapeutic guidance in monitoring or treating HIV infected individuals. We recognize that the mutations listed in Tables A and B (see below) are associated with HIV drug resistance. Other mutations, including those listed in Tables C-E (see below), are suspected of being associated with HIV drug resistance, but their significance has not been widely accepted.

HIV Drug Resistance Assays for which clinical trials have shown a clear medical benefit need further validation by analytical studies only to the degree necessary to characterize the scientific basis of the assay.

In this document, we describe two pathways for you to seek clearance of your assay as a Class II device for detecting HIV mutations. The first pathway is based on your

demonstrating rigorously the analytical sensitivity of your test for mutations in Tables A through E, below. The second pathway allows you to perform a less rigorous demonstration of the analytical sensitivity of your test for the mutations listed in Tables A-E, provided that data from clinical studies give evidence that use of the test will provide a medical benefit. We recognize that as the field progresses, additional mutations may become widely recognized as clinically significant. As advances are made in science and technology, we will amend the guidance as appropriate.

This guidance does not supersede other publications, but provides additional clarification on the information you should provide to us for review. You may refer to 21 CFR 807.87 for information that you must include in a premarket notification 510(k) for a medical device and to 21 CFR 809.10 for information about the labeling of in vitro devices. You are responsible for complying with the 21 CFR Part 820, Quality System Regulation for Class II or Class III devices, which includes Design Controls and Corrective and Preventive Action.

D. Regulatory Jurisdiction

Devices approved after 1976 for which there is no predicate device are generally classified as class III devices. However, FDA may reclassify such devices by using appropriate mechanisms. We believe that HIV Drug Resistance Assays may be suitable for reclassification and regulation as class II devices subject to special controls. This draft guidance document may serve as a special control if we reclassify these devices to class II. You should contact the Division of Blood Applications at CBER (301-827-3524) for information on filing your submission and for any questions you may have.

Analyte Specific Reagents

This guidance applies to HIV Drug Resistance Assays, but not to Analyte Specific Reagents (ASRs). ASRs are substances that are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens. ASRs are Class III devices when they are intended as a component in a test for use in the diagnosis of a contagious condition, such as HIV. We consider commercially distributed ASRs used in genotyping systems to detect HIV mutations to be class III devices requiring premarket approval.

II. SCIENTIFIC AND CLINICAL BACKGROUND

Current standards for care of HIV infected patients rely heavily upon tests for viral load (copies/ml of virus in serum/plasma). Therapy is designed, in part, to decrease the viral load as much as possible (generally, to below detectability). High viral loads and/or viral load rebound during HAART (Highly Active Anti-Retroviral Therapy - currently three and even four drug regimens) is taken as an indication of treatment failure. One of the most common causes of treatment failure is the existence or emergence of virus species resistant to the drugs included in the regimen (Ref. 1). Assays have been developed to identify the genotypes of virus present in infection. These assays identify the nucleic acid sequences in specific portions of the HIV genomes [e.g., the protease (PR), and reverse transcriptase (RT), genes] that make up the viral population in a patient and are being used to guide treatment choices for patients. However, multiple problems are associated with the use of such genotyping assays. Generally they detect only the most prevalent members of the viral "swarm." So called "archived" species, which may have accumulated during development of resistance to previous anti-retroviral therapy and which may be remnant at significant levels, may be undetectable by genotyping assays. Furthermore, the correlation between viral sequence and clinical resistance may be poorly determined. Some "resistance" mutations may appear early in anti-retroviral therapy and may indeed herald the onset of resistance, but may have only minimally detectable effects in various in vitro drug resistance assays. Absolute IC50 or IC90 (50% or 90% inhibitory concentration) levels may vary from assay to assay and may be difficult to relate to in vivo drug levels. Cross resistance, interference and the existence of phenotypes based on changes at multiple viral genetic loci may further confound the significance of genotyping data (Ref. 2).

We are providing this guidance to help you to assure the reliability of drug resistance genotype assays for recognized mutations and to show you how such assays may be developed and verified for review by the FDA as Class II medical devices. We are willing to work with you to determine the correlation between use of the assay and benefit to the patient for mutations that are currently not generally recognized as being associated with HIV resistance to anti-retroviral drugs.

III. DATA CONSIDERATIONS

We may request data and statistical analysis in premarket notification submissions to market in vitro diagnostic devices. The types of data and analysis that we may request depend on the technological characteristics of the new device, how you intend the device to be used, and the claims you intend to make for it. You can establish the performance of the device by comparison to any legally marketed medical device with the same intended use and/or by other studies to determine the operating characteristics of the device.

Generally, drug resistance genotype tests have two critical components: (1) the assay that determines and reports the genotype; and (2) the interpretation algorithm, which is a data analysis method by which the genotype is interpreted to predict the phenotype of the infecting viral swarm. Both components contribute to overall assay performance.

You may use a minimal interpretation algorithm outlined in this document (Tables A and B, below) or you may submit data supporting the use of additional interpretation rules.

You should submit:

- scientific data to support the performance characteristics of the device;
- documented protocols for in house and external testing;
- test results including analyses and conclusions; and
- summaries of results and explanations of unexpected results, charts (scatter grams, histograms, etc.).

You should submit unprocessed laboratory data, including line listings and actual data sheets when we specifically request them.

While it is not possible to list all scientific data that you might need to submit for a particular device, we have outlined the types of data and/or performance characteristics that you should consider including in a 510(k) submission to characterize the performance of the HIV Drug Resistance Genotype assay.

We believe that certain mutations in the HIV genome have been proven to be associated with viral resistance to specific anti-retroviral drugs used to treat HIV infection. We recognize that other mutations of interest in the HIV genome have not been proven to be associated with viral resistance to certain anti-retroviral drugs. We believe that you need only to provide analytical data demonstrating the ability of your tests to detect mutations in both these categories. However, we realize that the existence of supportive clinical trial data can increase confidence in the ability of an assay to be of benefit to the patient and that this increased confidence may reduce the nature and extent of analytical studies to assure assay effectiveness. For this reason, we are willing to accept less extensive analytical data on "established" and "implicated" mutations when supporting clinical trial data is submitted.

Thus, this document provides for two pathways to 510(k) clearance. In the first option, you may obtain clearance with extensive analytical data alone. In the second option, when you submit strongly supportive clinical data from trials using the investigational assay you may elect to submit limited analytical data. However, reliance on clinical data and less extensive analytical data may limit the claims of intended use set out in the labeling. (See Section VI.B of this guidance.)

A. Performance of the Interpretation Algorithm

1. Validation of Phenotypes Predicted by Genotyping: In Vitro Studies

You should support any phenotypic prediction based on genotypic information either by reference to Tables A and B, below, or by additional analytical verification studies. You should include in your verification studies for mutations not listed in Tables A or B in vitro assays measuring the binding of the active form of the anti-retroviral inhibitor to its target substrate and in vitro viral replication assays (including determination of the effect of the given genotype on IC_{50} or IC_{90}). You should further support phenotypic predictions not

listed in Tables A or B by including clinical data, as outlined below in III.A.2. You may submit verification studies derived in whole or in part from data previously published in peer reviewed journals. When relying upon previously published data, you should provide legible copies of all publications used to support your claims, together with individual summaries, in English, of individual publications and an overall summary of all the literature cited

2. Verification of Phenotypes Predicted by Genotyping: Clinical Studies

You should verify any phenotypic prediction not listed in Tables A or B, by clinical studies that correlate the existence and/or appearance of the corresponding genotype in patients with the existence and/or development of partial or complete resistance to specific therapy. Patient viral burden should be determined throughout these studies. You may submit verification studies derived in whole or in part from data previously published in peer reviewed journals. When relying upon previously published data, you should provide legible copies of all publications used to support your claims, together with individual summaries, in English, of individual publications and an overall summary of all the literature cited.

B. Performance of the Assay in Determining Genotype

1. Analytic Sensitivity

a. You should test panels of virions from cloned virus or patient specimens containing known, common single-locus mutations (e.g., a particular amino acid or sequence at a particular locus) or multiple-locus mutations, to determine analytic sensitivity.

- In the Specific Performance Characteristics section of the package insert, you should list all mutations which you can demonstrate the assay successfully detects according to the criteria laid down in this section and the immediately following section of this guidance document (III.B.1, a & b). Throughout this guidance document, we will refer to this list as the "Fully Verified Performance" list.
- You should test all mutations that will be listed in the Fully Verified Performance list of the package insert (see VI.B, below) as well as all mutations listed in Tables A and B. We may clear submissions that present data from an incomplete subset of the studies described in this and the immediately following sections (III.B.1.a & b) specifically limiting the Fully Verified Performance list to a subset of mutations for which sufficient analytical data has been provided, if data from clinical trials (see section III.E) using the assay support the clinical utility of the assay.
- You may test multiple related or unrelated mutations together in the context of a single genomic clone. In cases where codon degeneracy (i.e., alternative sequences coding for the same amino acids) allows

different sequences to code for identical amino acid mutations, you may test any single nucleic acid sequence that codes for the amino acids in question. However, in the package insert, you must list all relevant potential codons (i.e., alternative sequences coding for the same amino acid) that were not specifically tested.

- You should submit to FDA the identity of any specific mutations at the nucleic acid level that are known to be unusually difficult to sequence if they contribute to the interpretation algorithm you use in reporting assay results.
- You may construct panels by spiking methods, using well-characterized HIV-1 clones.
- You should test each mutation at least ten (10) times in these studies, at or near the lowest viral level that the assay can reliably detect. When using a clinical specimen for these studies, you should determine the sequence of the specimen's viral "swarm" by sequencing at least 10 molecular subclones.
- You should use three different lots of the assay in these analytical sensitivity studies.
- You should include in your submission a brief study description and well-organized data presentation including:

i. the identity and number of loci tested;
ii. the number of times each was tested;
iii. the genetic context in which each was tested;
iv. the viral load tested (copies/ml);
(1) the overall sensitivity (number correctly identified /total); and

(2) a summary of lot distribution over the studies.

b. You should also test panels that include clones with known, preferably common, multiple mutations (i.e., multiple mutations which need to be simultaneously present in order to allow resistance predictions).
You may obtain these clones from patients or by using site directed mutagenesis.
You should test each of these clones at least ten times, using three different lots of the assay, at clinically relevant viral loads.

c. You should clearly characterize the clones particularly with respect to the identities of the mutations in each clone.
You should conduct studies similar to those described in III. B. 1. a & b, immediately above, to show that the assay can detect all mutations listed in Tables C, D & E (below), as well as all mutations that are used in the interpretation algorithm.
For these studies, you should demonstrate the ability of your assay to detect at least one common mutation codon (at the corresponding locus) for each mutation listed and at one level of virus (copies per ml.), as specified in this paragraph.
You should demonstrate that mutations listed as "Primary" in these tables can be routinely detected at viral levels within four fold of the minimum levels for which a claim is sought.

You may demonstrate the detectability of "Secondary" mutations at any level within the useable range of the assay.
For mutations listed in Tables C – E, or other mutations used in the interpretation algorithm that are also listed in Tables A or B or in the Fully Verified Performance list, you should perform the studies described above in sections III.B.1. a. & b.
We may clear your submissions presenting data from an incomplete subset of the studies described in this section (III.B.1.c.) if your data from clinical trials (see section III.E) using the investigational assay support the clinical utility of the assay. However, in such cases, we may require precautionary labeling in the Limitations for Use section of the package insert indicating which mutations have been incompletely tested and verified.

d. Generally, assays should correctly identify the amino acids at all codons in Protease and Reverse Transcriptase known or suspected to be involved in conferring drug resistance. We will consider for clearance on a case by case basis assays that fail to do so if they serve a specific and demonstrable public health need. In such cases, labeling for the assays should describe the device's more limited uses. A precautionary statement warning that the device has reduced sensitivity may be necessary, even if the codons that the device fails to read are only suspected of conferring resistance and are not fully verified.

2. Range of Detectability

You should define the overall plasma/serum concentration of virus (viral burden) at which these tests are effective. The assay should be effective at a viral burden that is clinically relevant.
You should determine assay performance (sensitivity and specificity for specific genotypes) over the entire range of the assay, both with respect to overall viral levels (copies/ml) and with respect to the percent representation of specific mutations (e.g., 25% of total).

In general, it is important to determine assay performance as overall levels and/or mutant proportions decrease.
Although you should determine the sensitivity and specificity at all loci specifically listed in the Fully Verified Performance list of the package insert (see VI.B, below), you need to fully evaluate only a representative set of 30 total loci for each parameter (viral level & mutant proportion) according to the criteria described in these sections (III.B.2.a, b & c). These 30 loci may consist of any of the loci listed in Tables A or B or in the Fully Verified Performance list.
We may clear submissions lacking studies described in these sections (III.B.2.a, b & c), and limit the Fully Verified Performance list to a subset of the mutations in Tables A and B for which you have provided sufficient analytical data, if there is strongly supportive data from clinical trials using the investigational assay.

a) Using the minimal proportions of mutant species in the range of detectability, you should determine assay performance at two-log (or smaller) intervals above the minimal viral levels in the range of detectability and up to the maximum level in the range of detectability. Similarly, you should determine assay performance at half-log intervals (or smaller) below (and including) the minimal level in the range of detectability, and down to 1.0 log below minimum. Thus, if 30% is the minimal proportion of mutant species that the assay can reliably detect and 1000 copies/ml is the minimal viral level at which the assay can reliably obtain sequences, you should test the following levels of virus mixtures (in copies/ml, containing 70% wild type and 30% mutant): 100; 300; 1000; 100,000 (or less); 10,000,000 (or less, but not to exceed 100 times the next lower level tested, nor to exceed the useable range of the assay).

b) Using the minimal viral levels in the range of detectability, you should determine assay performance at approximately 100%, 80% and 50% of the minimal mutant proportions in the range of detectability, as well as at least two higher levels selected to be equally spaced (linearly) between the minimal proportion and 100% proportion. You should also test mutant at a proportion of 100%. (Thus, for example, if you seek a claim for 25% mutant the following proportions of mutant would be tested: 100%, 75%, 50%, 25%, 20% and 12.5%.)

c) You should report the mutant/wild type ratios tested and sensitivity at each level.

3. Precision

For assays which claim to determine the quantitative levels or proportions of viral mutants (rather than just presence or failure to detect), precision studies should define the coefficients of variation for the HIV resistance assay within one experiment using one product lot and also across three product lots. You should include in your study at

least 20 10-aliquot sample sets (20 different validated mutations at 20 different loci, 10 replicates, for each lot). You should do your studies at the lowest level in the range of detectability and additionally at higher levels, at your discretion.

4. Reproducibility

You should determine assay reproducibility testing three lots at different sites, on different days, and by different investigators. You should analyze samples in triplicate, including a subset of mutations for which claims are sought.

5. Lot acceptance testing

You should perform lot acceptance testing to assure adequate performance of each lot of assay produced. Lot acceptance testing should include data indicating adequate performance with panel members at the lowest levels/proportions in the range of detectability. All amino acid mutations for which a claim is sought should be tested, at least singly.

6. Specificity

During the course of analytical sensitivity studies, we expect that many defined analytes with various combinations of wild type loci and resistance mutations will be tested. You should accumulate, analyze and report data from these experiments concerning the non-specificity of the assay (i.e., how often the assay reports an incorrect result at wild type loci).

7. Assay Interference

Most assays are subject to interference from specific components. These components may be introduced during sample collection and handling or they may be present in the patient as a result of the patient's therapy or condition. You should determine the effects on the assay of a variety of substances and conditions that are likely to cause interference. You may test for interference using spiking methodology in addition to testing original clinical specimens. Some conditions that we may expect to cause interference include:

a. Other infections including HIV-2, Human T-cell Lymphotrophic Virus Type I/II (HTLV-I/II), Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), yeast infections, pneumocystis, *M. tuberculosis, M. Avium and M. intracellulare*;

b. Samples collected in various anticoagulants, or other collection media;

c. Hemolyzed, icteric, lipemic, and bacterially contaminated samples;

d. Chemicals, drugs, heated, and detergent treated samples;

e. Samples subjected to multiple freeze thaw cycles;

f. Fresh vs. frozen samples, serum vs. plasma, and single specimen vs. plasma pool;

g. Samples from patients with autoimmune diseases including Systemic Lupus Erythematosus (SLE), Anti-Nuclear Antibodies(ANA), Rheumatoid Arthritis mixed cryoglobulinemia;

h. Nucleic acid based drugs, metabolites and binding substances, particularly those known or suspected to have inhibitory effects on reverse transcription.

i. Drugs commonly used for treatment of opportunistic infections associated with HIV, including ganciclovir, foscarnet, anti-mycobacterials, ribavirin and alpha-interferons.

8. Reagent Characterization

You should characterize the nucleic acid sequences (primers, probes, etc.), capture agents, enzymes, controls and calibrators used in the assay. You should describe the rationale and methods used to qualify each lot of critical components. Please refer to the December 1999 "Guidance In the Manufacture and Clinical Evaluation of *In Vitro* Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Virus Types 1 and 2" section III. for further guidance. (Ref. 4)

9. Sample collection and handling conditions

If two or more types of specimens are recommended for testing, you should determine the performance characteristics for each type of specimen, unless you can demonstrate that different specimen matrices, anticoagulants, etc., do not affect assay results differentially.

C. Stability

You should determine the stability of critical components (nucleic acid sequences, capture agents, enzymes, controls, calibrators, clones or transcripts, as applicable) and should maintain files with the raw data for inspection by FDA. You do not have to submit this data to us unless specifically requested to do so, but may provide the data in summary form.

D. Assay Performance on Clinical Samples

1. Sensitivity on Clinical Samples.

In your Sensitivity studies you should include a panel of 50 unspiked, repository specimens selected to have viral loads between the lower limit of detection (LoD) and fourfold of the LoD (LoD X 4) whose genetic make up has been determined by molecularly subcloning and sequencing 40 subclones each (or by equivalent

techniques). Selection of the clinical specimens should be random for characteristics other than viral load. You should determine the performance of the assay in these studies for all mutations listed in Tables A and B. To test the performance on Table A and B mutations not adequately represented in the randomly selected panel of 50 clinical samples, you should acquire specimens which do represent them and test them both neat and diluted to between LoD and LoD X 4 copies per ml. We will consider exceptions in cases where certain specific mutations are very rare and unlikely to be obtained. We may clear submissions lacking the studies described in this section (III.D.1) with the Fully Verified Performance list limited to the subset of the mutations in Tables A and B for which sufficient analytical data has been provided, but only if there is strongly supportive data from clinical trials using the investigational assay included in the submission.

2. Population Sensitivity Studies

You should also determine how frequently, in a target population, the assay gives interpretable data. You should include in such studies 100 random clinical specimens with viral loads distributed throughout the clinically relevant, useable range of the assay, including a substantial number within the range of approximately LoD to LoD X 4.

3. Specificity on Clinical Samples

During the course of the clinical sensitivity testing, described immediately above in III.E.2, you should test a variety of defined samples, representing various combinations of wild type loci and resistance mutations. You should accumulate, analyze and report data from these experiments concerning the non-specificity of the assay (i.e., how often the assay reports an incorrect result at wild type loci).

4. Reproducibility on Clinical Samples

You should determine clinical reproducibility using these repository specimens (as described in paragraph III.D.1, above). Each specimen should be determined in triplicate, on different days, at different sites, by different investigators and using three different lots.

E. Clinical Trial Data Supporting Efficacy

You do not need to submit clinical trial data demonstrating the efficacy of your assays for clearance when you submit complete sets of analytical data, as described in sections III.B.1 & 2 and III.D.1, above. However, you may lessen the nature and extent of analytical studies, as described in sections III.B.1 & 2 and III.D.1, above, if you submit supportive data from clinical trials directly demonstrating that the use of your assay results in clinical benefit and if you are willing to accept specific restrictions on certain claims made in the labeling. An example of an appropriate clinical trial would be a study comparing use vs.

non-use of the investigational assay, measuring clinical endpoints. Clinical endpoints could be AIDS defining events, death or acceptable surrogate markers, such as viral burden. Thus, we have identified two tracks for clearance, one requiring extensive analytical data and the other requiring limited analytical data in combination with clinical trials and entailing specific limitations on claims made in the labeling. A summary chart that highlights the differences between these two tracks is presented in Table F.

F. Modifications of Criteria for Special Purpose Assays

We may clear "special purpose" assays with limited claims for subsets of the mutations listed in Tables A and B, if you successfully demonstrate that doing so would benefit the public health.

Table A (Ref. 3)

Mutations Recognized to Confer Clinical Resistance to Reverse Transcriptase Inhibitors

Mutation	Resistance Profile	Interpretation
M41L	ZDV	Confers resistance in combination with other ZDV mutations
A62V	Multi-NRTI	Uncommon, only confers resistance in combination with F75I, F77L, F116Y, and/or Q151M
K65R	DDC, DDI, ABC	Confers resistance to DDI and ABC usually in combination with other mutations. As a single mutation may cause resistance to DDC
D67N	ZDV	Confers resistance in combination with other ZDV mutations
S68G	Multi-NRTI	Uncommon but usually confers resistance in combination with A62V, F77L, F116Y, and/or Q151M
T69D	DDC	As a single mutation may confer resistance
69ins	Multi-NRTI	Confers resistance usually in combination with ZDV resistance mutations (M41L, D67N, K70R, L210W, T215Y/F, K219Q/E)
K70R	ZDV	Confers resistance in combination with other ZDV mutations
L74V	DDI, DDC, ABC	As a single mutation may cause clinical resistance to DDI and DDC, additional mutations may be required for ABC
V75I	Multi-NRTI	Uncommon, only confers resistance in combination with A62V, F77L, F116Y, and/or Q151M
F77L	Multi-NRTI	Uncommon, only confers resistance in combination with A62V, F75I, F116Y, and/or Q151M
L100I	NVP, EFV	Often found in combination with other mutations
K103N	NNRTI (all)	As a single mutation confers resistance
V106A	NVP, DLV	As a single mutation confers resistance
V108I	NVP, EFV	Often found in combination with other mutations
Y115F	ABC	Confers resistance in combination other ABC mutations or with ZDV mutations
F116Y	Multi-NRTI	Uncommon, only confers resistance in combination with A62V, F75I, F77L, and/or Q151M
Q151M	Multi-NRTI	Usually confers resistance in combination with A62V, F75I, F77L, F116Y
Y181C/I	NVP, DLV	As a single mutation confers resistance
M184 I/V	3TC, ABC, ddC, DDI	As a single mutation confers resistance to 3TC and ddC, the addition of other mutations may be required for clinical resistance to ddI or ABC
Y188C/L	NNRTI (all)	As a single mutation confers clinical resistance
L210W	ZDV	Confers resistance in combination with other ZDV mutations
T215Y/F	ZDV	Confers resistance in combination with other ZDV mutations
K219Q/E	ZDV	Confers resistance in combination with other ZDV mutations

*Multi-NRTI refers to zidovudine (ZDV), didanosine (DDI), zalcitabine (DDC), abacavir (ABC), stavudine (D4T) **All NNRTI = nevirapine (NVP), delavirdine (DLV), efavirenz (EFV)

Table B (Ref. 3)

Mutations Recognized to Confer Clinical Resistance to Protease Inhibitors

Mutation	Resistance Profile	Interpretation
D30N	NFV	As a single mutation confers resistance to NFV
M46I	ALL PIS	Confers resistance in combination with other mutations associated with clinical resistance
G48V	SQV	Confers resistance in combination with other mutations associated with clinical resistance
I50V	APV	Confers resistance usually in combination with other mutations
I54V	ALL PIS	Confers resistance in combination with other mutations associated with clinical resistance
V82 (A/F/T/S)	RTV, IDV, LPV/RTV, NFV, SQV	More strongly associated with IDV, RTV, and LPV; Confers resistance usually in combination with other mutations
I84V	ALL PIS	Confers resistance usually in combination with other mutations
N88D	NFV	
L90M	ALL PIS	More strongly associated with SQV or NFV but in combination with other mutations may confer resistance to all PI

ALL PIS = APV (amprenavir), IDV (indinavir), LPV/RTV (lopinavir/ritoanvir), NFV (nelfinavir), SQV (saquinavir), RTV (ritonavir)

Table C (Ref. 3)

Mutations in the Protease Gene Selected by Protease Inhibitors

(Primary mutations generally cause decreased inhibitor binding and are the first mutations selected during therapy with the associated antiretroviral. Secondary mutations may also contribute to drug resistance, although they may have less direct effect on inhibitor binding in vitro than primary mutations -Ref. 3).

Drug	Degree	Associated Mutations
Indinavir	Primary	M46I; V82A, or F, or T, or S
	Secondary	L10I, or R, or V; K20M, or R; L24I; V32I; M36I; I54V; A71V, or T; G73S, or A; V77I; I84V; L90M
Ritonavir	Primary	V82A, or F, or T, or S
	Secondary	K20M, or R; V32I; L33F; M36I; M46I, or L; I54V, or L; A71V, or T; V77I; I84V; L90M
Saquinavir	Primary	G48V; L90M
	Secondary	L10I, or R, or V; I54V, or L; A71V, or T; G73S; V77I; V82A; I84V
Nelfinavir	Primary	D30N; L90M
	Secondary	L10F, or I; M36I; M46I, or L; A71V, or T; V77I; V82A, or F, or T, or S; I84V; N88D
Amprenavir	Primary	I50V; I84V
	Secondary	L10F, or I, or R, or V; V32I; M46I; I47V; I54V

Table D (Ref. 3)

Mutations in the Reverse Transcriptase Gene Selected by Nucleoside Reverse Transcriptase Inhibitors

(Primary mutations generally cause decreased inhibitor binding and are the first mutations selected during therapy with the associated antiretroviral. Secondary mutations may also contribute to drug resistance, although they may have less direct effect on inhibitor binding in vitro than primary mutations -Ref. 3).

Drug	Degree	Associated Mutations
Zidovudine	Primary	K70R; T215Y, or F
	Secondary	M41L; D67N; L210W; K219Q
Stavudine	Primary	V75T
Didanosine	Primary	L74V
	Secondary	K65R; M184V, or I
Zalcitabine	Secondary	K65R; T69D; L74V; M184V, or I
Lamivudine	Primary	E44D; V118I; M184V, or I
Abacavir	Primary	K65R; L74V; M184V
	Secondary	M41L; D67N; K70R; Y115F; L210W; T215Y, or F; K219Q
Multi-nRTI Resistance-A	Primary	Q151M
	Secondary	A62V; V75I; F77L; F116Y
Multi-nRTI Resistance-B	Primary	T69S and 2 amino acids encoded by an insertion between RT codons 69 and 70 (69 Insertion)
	Secondary	M41L; A62V; D67N; K70R; L210W; T215Y, or F; K219Q

Table E (Ref. 3)

Mutations in the Reverse Transcriptase Gene Selected by Non-nucleoside Reverse Transcriptase Inhibitors

(Primary mutations generally cause decreased inhibitor binding and are the first mutations selected during therapy with the associated antiretroviral. Secondary mutations may also contribute to drug resistance, although they may have less direct effect on inhibitor binding in vitro than primary mutations -Ref. 3).

Drug	Degree	Associated Mutations
Nevirapine	Primary	K103N; V106A; V108I; Y181C, or I; Y188C, or L, or H; G190A
	Secondary	L100I
Delavirdine	Primary	K103N; Y181C
	Secondary	P236L
Efavirenz	Primary	K103N; Y188L; G190S, or A
	Secondary	L100I; V108I; P225H

Table F

Highlights of the different requirements required by the "Analytical-Data-Only" and "Clinical Trial" tracks to clearance.

[Only differing requirements are listed. See the body of the document for full requirements.]

Section	"Analytical Data-Only" Track	"Clinical Trial Track"
III.E	No clinical trial data	Clinical trial data demonstrating use of sponsor's assay has benefit defined by clinical progression or surrogate markers.
III.B. 1 a & b	Stringent analytical sensitivity on cloned isolates or clinical samples, covering all mutations in Tables A & B.	Stringent analytical sensitivity studies on a subset of the mutations in Tables A & B. FDA may require mutations in Tables A & B not covered by these studies to be omitted from the list of mutations in the Fully Verified Performance list.
III.B. 1.c	Less stringent analytical studies on all mutations in Tables C, D & E.	Less stringent analytical studies on mutations in Tables C, D & E are desirable, but not required. FDA may require incompletely verified mutations to be listed in the Limitations for Use section.
III.B. 2. a &b	Titration of assay performance across various viral levels and wild type/mutant proportions on a subset of 30 of the mutations listed in Tables A&B and the Fully Verified Performance list.	FDA may require mutations in Tables A & B not covered by studies on the titration of assay performance (across various viral levels and wild type/mutant proportions) to be omitted from the Fully Verified Performance list.
III.D. 1	Analytical sensitivity on a panel of 50 clinical specimens (characterized by sequencing 40 subclones each), covering all mutations in Tables A & B.	Full set of 50 panel members not required. FDA may require mutations in Tables A & B not covered by these studies to be omitted from the Fully Verified Performance list.
VI.B	All mutations in Tables A and B may be listed in the "Indications for Use" statement.	The only mutations listed in the "Fully Verified Performance list will be those for which the sponsor has submitted full analytical data, equivalent to that required in the "Analytical-Data-Only" track.

IV. OTHER CONSIDERATIONS

A. Design Controls.

You should consult the FDA document "Design Control Guidance for Medical Device Manufacturers" (March 11, 1997); and the regulations in 21 CFR Part 820 to assure adequate design of the entire system, from sample acquisition through data interpretation and reporting at sites of intended use.

B. Statistical Methods.

You should ensure that all statistical methods in a 510(k) are appropriate for the study protocol, type of data collected and intended use of the device. You should select statistical methods from recognized sources and properly reference them in the submission. We encourage you to discuss these aspects with us during the planning phases of your studies.

C. Devices used for generating data for submission.

You should perform all studies either with a product which is representative of the final product that will be marketed or one that can be related to that product through concurrent testing.

D. Instruments.

You should make information about instruments that are dedicated components of the assay part of the submission. You should describe the function, operating characteristics, and manuals for each instrument.

E. Pre-submission meetings.

We encourage you to meet with us prior to filing your submission to clarify current FDA policy and to resolve questions not met by current guidance.

V. PRODUCT MODIFICATION

When a product has been cleared for marketing through a 510(k) mechanism, and modifications are made to the product that alter its indications for use and/or change the fundamental scientific technology, you must submit a new 510(k) for the change and obtain clearance to market the changed device. Specific examples of when a new 510(k) should be filed include, but are not limited to, new labeling for genotypic prediction of phenotypic resistance for new anti-viral drugs, new labeling for newly discovered mutations or mutations with newly documented phenotypes, or material changes in the interpretation algorithm. You should consult the Office of Device Evaluation's Memorandum entitled "Deciding when to submit a 510(k) for a Change to an Existing Device," January 10, 1997, (Ref. 5) if you are considering a change to your product.

VI. LABELING

A. Intended Use

- The intended use statement should read, "...for use in detecting HIV genomic mutations that confer resistance to specific types of anti-retroviral drugs, as an aid in monitoring and treating HIV infection."
- You should not mention specific mutations or loci in the intended use statement.
- You should label your product in accordance with 21 CFR 809.10 including: the types of samples (serum, plasma, cells, etc.); method of collection (anticoagulants); the analyte to be studied (DNA or RNA); the effective range of concentration of virus detectable; the viral subtypes for which a claim is sought; and the clinical situations in which use of the assay is appropriate.

B. Specific Performance Characteristics

- You should include in this section of the package insert the "Fully Verified Performance" list. This should be a list of all mutations for which full analytical studies have been successfully completed according to the requirements of sections III.B.1 a & b of this guidance document.
- You may include in this list mutations not listed in Tables A and B if you perform full analytical studies on them as described in sections III.B.1 a & b and, if you submit data that verifies their clinical significance to the extent that it justifies their use in the interpretation algorithm without associated precautionary labeling or disclaimers.

- We may reduce the scope of this list if you have not successfully completed all of the studies recommended in sections III.B.2 and III.D.1 of this guidance document.

C. Directions for use

Interpretation and Reporting of Assay Results

You should provide, in the package insert, an interpretation algorithm to translate raw data into indications of drug resistance profiles. You should clearly describe the entire algorithm. At a minimum, the information provided in Tables A and B may serve as an interpretation algorithm. You may incorporate in the assay interpretation algorithm all mutations listed in Tables A and B, above, without modification, together with their listed interpretations. You may incorporate in the assay interpretation algorithm mutations not listed in Tables A or B, above, or modifications of interpretations listed in Table A or B, above, if you present and summarize the data supporting each such proposed interpretation in the submission. You may include in such supporting data, original data, or data cited from peer reviewed literature as is described in section III.A.1 & 2. In this section of the labeling, you should list any interpretation rule that is used in the algorithm, but that is not specifically listed in Tables A or B, or that relies upon a mutation that has not been otherwise qualified to be included in the Fully Verified Performance list. In some cases, it may be appropriate for you to include in the interpretation algorithm an incompletely verified interpretation rule if the package insert identifies that interpretation rule and states (1) that incompletely verified data were used to support it; and (2) that the clinical significance of the interpretation rule has not been fully verified. Furthermore, you should summarize the justification for any such rule in the package insert, with references to the supporting literature and/or summaries of original, submitted data, as appropriate.

D. Limitations for Use

You should prominently list in this section, mutations in Tables A-E for which you have not performed the analytical studies outlined in this guidance document.

You should also prominently list in this section, any mutations used in the interpretation algorithm for which you have not performed analytical studies outlined in this guidance document.

You should indicate the approximate minimum detectable proportion of virus in the total population (e.g., a mutant at a level of 25% against a background of 75% wild type can be detected, but the same mutant at a level of 10% is not detected). You should also indicate the approximate minimum viral level (copies per ml.) at which the assay can give reliable data.

The limitations section should also describe any interfering substances, conditions, or other factors that can affect the performance characteristics of the assay.

VII. REFERENCES

1. Perrin, L. & Telenti, A (1998); Science 280, 1871-1873.

2. Hirsch, M.S. et al. (1998); JAMA 279, 1984-1991.

3. Hirsch MS, Brun-Vezinet F, D'Aquila RT, Hammer SM, Johnson VA, Kuritzkes DR, Loveday C, Mellors JW, Clotet B, Conway B, Demeter LM, Vella S, Jacobsen DM, Richman DD "Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel." JAMA. 2000 May 10, 283(18), 2417-26.

4. Guidance In the Manufacture and Clinical Evaluation of *In Vitro* Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Virus Types 1 and 2, December 1999: http://www.fda.gov/cber/gdlns/hivnas.pdf.

5. "Deciding when to submit a 510(k) for a Change to an Existing Device," January 10, 1997, Office of Device Evaluation, CDRH, FDA. http://www.fda.gov/cdrh/ode/510kmod.html.

6. "Design Control Guidance for Medical Device Manufacturers", March 11, 1997, Office of Device Evaluation, CDRH, FDA. http://www.fda.gov/cdrh/comp/designgd.pdf.

Stanford HIV Drug Resistance Database

Queries - PI Susceptibility Data

Mutations	N88T	**References**	3
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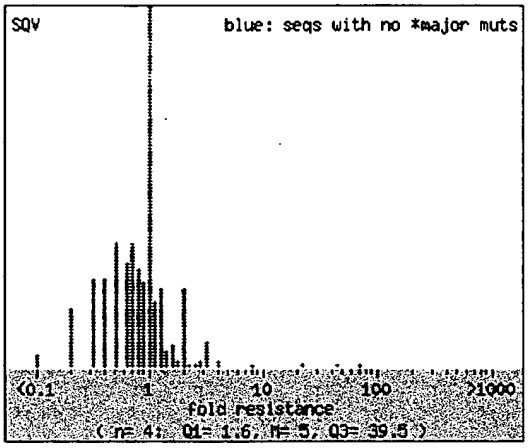
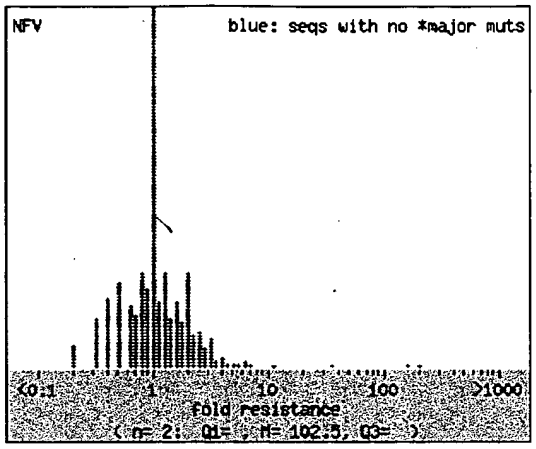
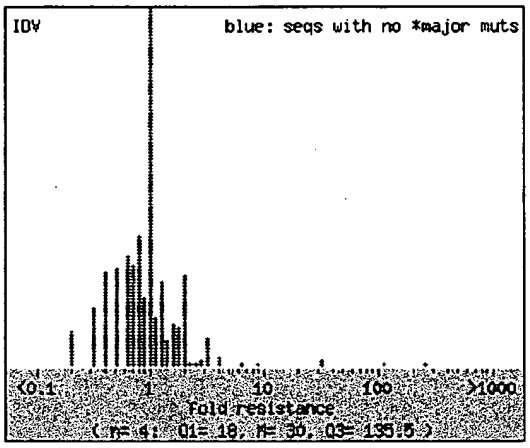
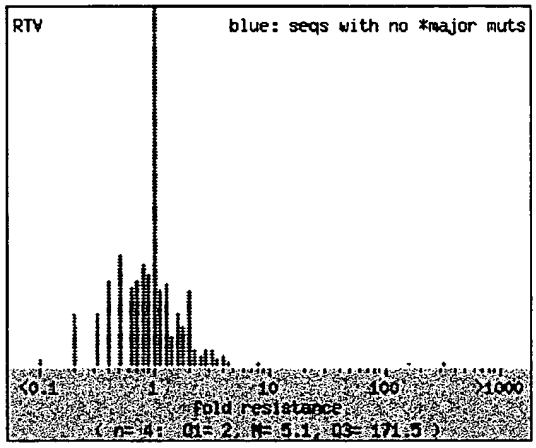
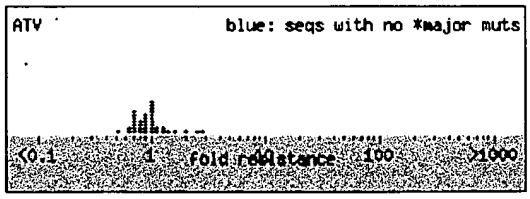
Assay	ANY	**Results**	18
WithNoOtherMajorMuts	No		

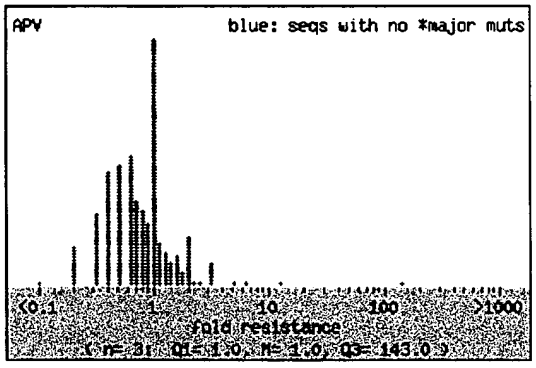


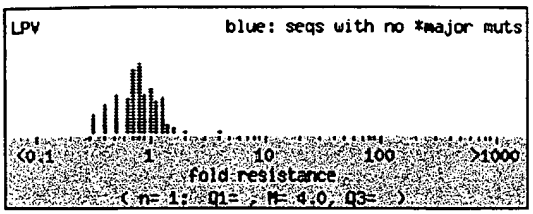












* 8,24,30,32,46,47,48,50,53,54,73,82,84,88,90
n: number of phenotype results, Q1: 1st quartile, m: median, Q3: 3rd quartile

Page 1 from Result 1 to Result 18 from Total 18 Phenotype results

Author (yr)	Type	Isolates	Major	Minor	Other	Method	Drug	Fold
Condra (1996)	Clinical	ptOW48	M46I, I84V, N88T	L10I, L63P, V77I, I93L		Condra-96	APV	1.0
						Condra-96	IDV	>30.0
						Condra-96	RTV	2.0
						Condra-96	SQV	2.0
		ptOW60	M46I, I84V, N88T	L10I, L63P, V77I, I93L	I66V	Condra-96	APV	1.0
						Condra-96	IDV	>30.0
						Condra-96	RTV	>8.0
						Condra-96	SQV	8.0
Shafer (2000)	Clinical	CA9597	M46I, N88T	L10F, K20T, M36IM, L63P, A71T	I15V	Virologic	IDV	6.0
						Virologic	LPV	4.0
						Virologic	NFV	11.0
						Virologic	RTV	2.1
						Virologic	SQV	1.1
Beerenwinkel (2002)	Clinical	1798-RUH	I54V, V82F, I84X, N88T, L90M	L10I, K20R, M36I, L63P, A71T, I93L	I15V, L19I, E35D, I62M, I72V, I85X, R87T, Q92X, C95S, T96P, N98S	GermanNRC	APV	143.0
						GermanNRC	IDV	241.0
						GermanNRC	NFV	194.0
						GermanNRC	RTV	335.0
						GermanNRC	SQV	71.0

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